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Hyphal Elongation of *Glomus fasciculatus* in Response to Root Exudates†

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The spore germination rates on water agar of the vesicular-arbuscular mycorrhizal fungus *Glomus fasciculatus* were highest at water potentials of −4 to −6 bars. Root exudates from plants grown in a sterile nutrient solution, with or without phosphorus, did not affect germination. Root exudates collected from 2-, 4-, and 6-week-old *Trifolium repens* cv. 'Ladino' seedlings that were deprived of P enabled hyphal growth from germinated *Glomus fasciculatus* spores of 21.4, 14.7, and 7.6 mm, respectively. Hyphal elongation in the presence of exudates from plants grown with P, or in the absence of exudates, was negligible (<1 mm). Root P at 2 weeks was not significantly different between plants grown with and without P. There were no significant differences between the quantities of exudates from plants grown with or without P at 2, 4, and 6 weeks. The data suggest that it is the quality of exudates from plants experiencing P deprivation that is important in stimulating vesicular-arbuscular mycorrhizal hyphal elongation.

The influence of plant root exudates on soil microorganisms, "the rhizosphere effect," has been well recognized for some time (4, 14, 17, 21, 22, 32, 33, 36). More recently, several studies have been conducted to determine the influence of plant root exudates on vesicular-arbuscular mycorrhizal (VAM) colonization (2, 3, 7, 11–13, 15, 18–20, 24, 25, 27, 31). In addition, studies have specifically looked at the effect of plant age (19), plant species (39), and light (19, 34) on root exudation and in turn on VAM formation.

Until recently, most investigations have led scientists to conclude that P deficiency increases root exudation and that the quantity of exudates leaked into the rhizosphere increases VAM infection. However, Schwab et al. (38) found that there was no qualitative difference in the exudates from P-deficient plants as compared with those of nondeficient plants. These results, however, did not eliminate the possibility that some unknown component of the root exudates of P-deficient plants (6) produced at the onset of P deficiency could be a VAM growth factor. The presence or activity of such a factor has never been observed because experiments have not been designed for its demonstration. For example, Ratnayake et al. (31) collected exudates only once from 8- to 10-week-old sudangrass or sour orange seedlings. Graham et al. (13) collected exudates only once from 7- to 8-week-old sudangrass seedlings. Schwab et al. (37, 38) collected exudates only once from 6-week-old sudangrass and other seedlings. Since Rovira (32) has shown that root exudation decreases dramatically with plant age, a plant grown for 2 months without an external source of P is probably near death. If a VAM growth factor is produced at all, it should be looked for at the beginning of P deprivation; since it is probably transient, its presence should be monitored over several weeks. To our knowledge this has never been attempted. The objective of this study was to determine the effects of root exudates, produced by plants experiencing phosphorus deprivation, on VAM hyphal elongation.

MATERIALS AND METHODS

Plant material. White clover (*Trifolium repens* L. cv. 'Ladino') seeds were surface sterilized with 70% ethyl alcohol for 30 s followed by 0.1% $HgCl_2$ in 1 mM HCl for 5 to 7 min and washed exhaustively with sterile distilled water. The seeds were then placed on moist filter paper in sterile petri plates and incubated at 23°C in the dark for 2 days to allow germination. Seedlings were then taken from each petri plate and placed on moist cheesecloth in sterilized square glass staining dishes at 50 seedlings per dish. Each dish contained 100 ml of sterilized Hoagland nutrient solution, with or without phosphorus. The Hoagland nutrient solution contained the following (in 1 liter of glass-distilled water): KNO_3, 606.6 mg; $Ca(NO_3)_2 \cdot 4H_2O$, 656.4 mg; $MgSO_4 \cdot 7H_2O$, 240.8 mg; $NH_4H_2PO_4$, 110.0 mg; H_3BO_3, 2.86 mg; $MnCl_2 \cdot 4H_2O$, 1.81 mg; $ZnSO_4 \cdot 7H_2O$, 0.22 mg; $CuSO_4 \cdot 5H_2O$, 0.08 mg; H_2MoO_4, 0.02 mg; iron tartrate, 5.0 mg. The $NH_4H_2PO_4$ was omitted for phosphorus deprivation treatments. The pH was adjusted to 6.8, and then dishes were autoclaved at 121°C for 15 min. The dishes were then enclosed in sterile clear plastic bags and tightly sealed. The seedlings were grown under fluorescent lights (4.5 klx) with 16-h days.

The Hoagland solution was replaced every 7 days. Each treatment consisted of six replicate staining dishes that contained 50 seedlings. Parallel samples (five replicate plants per treatment) were taken at weekly intervals for 6 weeks to measure growth of whole plants, root systems, and root tissue phosphorus depletion rates.

Fungal material. The VAM species *Glomus fasciculatus* (Thaxt. sensu Gerd.) Gerd. and Trappe was grown in sorghum (*Sorghum vulgare*) pot cultures in the greenhouse for 4 months and stored at 4°C for at least 4 months before use. This VAM-infested soil was wet sieved, after which a modified centrifugation-flotation technique (30) involving 30% sucrose and several Ficoll solutions of various densities

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(10, 35, 45, and 60%) was used. Organic debris was carefully removed from the spore suspension by hand with a Pasteur pipette under a dissecting microscope. Fungal chlamydospores were then surface sterilized with a 1:1:1 solution of 2% (wt/vol) chloramine T, 0.02% (wt/vol) streptomycin sulfate, and sodium lauryl sulfate. After incubation under vacuum for 30 min, chlamydospores were washed with sterile distilled water (23). These chlamydospores were stored at 4°C for up to 5 days before use.

Spore germination. A medium designed for root organ culture (ROC) (16, 26), but modified to contain phosphorus, contain no phosphorus, or contain only water agar was used in this experiment. The ROC agar contained the following (in 1 liter of glass-distilled water): KCl, 65 mg; KNO_3, 80 mg; $Ca(NO_3)_2 \cdot 4H_2O$, 300 mg; $MgSO_4 \cdot 7H_2O$, 720 mg; $NaH_2PO_4 \cdot 2H_2O$, 10.7 mg; iron tartrate, 4.6 mg; $MnCl_2 \cdot 4H_2O$, 4.9 mg; KI, 0.75 mg; H_3BO_4, 1.5 mg; $ZnSO_4 \cdot 7H_2O$, 1.9 mg; $CuSO_4 \cdot 5H_2O$, 1.0 μg; $Na_2MoO_4 \cdot 2H_2O$, 0.17 μg; glycine, 3.0 mg; thiamine hydrochloride, 0.1 mg; nicotinic acid, 0.5 mg; pyridoxine, 0.1 mg; sucrose, 20 g; Bacto-Agar (Difco Laboratories), 8.0 g. The pH was adjusted to 4.9, and then the dishes were autoclaved at 121°C for 15 min. $NaH_2PO_4 \cdot 2H_2O$ was omitted for phosphorus deprivation treatments. To study the influence of water potential on VAM spore germination, we adjusted the osmotic potential of the media with sorbitol. Sorbitol was added to the ROC agar at a range of concentrations from 0 to 30 g/100 ml of water. Six replicate plates at each concentration were made. One plate was used to measure the water potential of the agar with the Wescor dewpoint hygrometer and C-52 sample chambers by a procedure modified from that of Nelsen et al. (29). Agar plugs (1-cm diameter) were taken from petri plates with a cork borer and placed in shallow sample chambers. Water potential was measured after at least a 2-h equilibration period. The other five replicate plates were inoculated with 20 surface-sterilized *G. fasciculatus* spores. Plates were incubated at 25 to 27°C in the dark, and percentage germination was determined after 15 days.

Exudate collection. At 2, 4, and 6 weeks exudates were collected from the roots of clover seedlings grown with or without phosphorus. At the end of each 2-week period, all plants were taken out of the nutrient solution and rinsed with sterile distilled water several times, and then 50 seedlings were placed in each staining jar containing sterile distilled water (50 ml) for 24 h. The distilled water in which seedlings were kept for 24 h was pooled for each treatment, filter sterilized (0.45-μm mesh), rotary evaporated at 40°C to 1/10 the original volume, filter sterilized again, and stored at 4°C. This exudate collection procedure was repeated at 4 and 6 weeks. Contamination checks were done once a week by plating out spent nutrient solution on water agar and potato-dextrose agar at the time of nutrient solution replacement. No contamination was observed.

Incorporation of exudates into agar medium. Root exudates, concentrated to 1/10 volume, from phosphorus-deprived and phosphorus-supplemented plants at 2, 4, and 6 weeks were added to ROC agar at a range of concentrations from 0.0 to 8.0 ml of exudate solution per 10 ml (total volume) of medium. Sorbitol was added to attain the optimum water potential of −5 bars for germination of *G. fasciculatus* spores, as determined previously. *G. fasciculatus* spores (30 per plate) were placed on plates amended with root exudate. All plates were incubated at 25 to 27°C in the dark. Germination and hyphal elongation were monitored at 5-day intervals. Germination is defined as a germ tube that is at least twice the diameter of the spore. Hyphal elongation data include the mean hyphal lengths of only those spores that germinated. This experiment was performed twice.

Quantification of exudates. In an additional replication of this experiment, the quantities of root exudates from 2-, 4-, and 6-week-old seedlings grown with and without P was determined after lyophilization. A VIRTIS model 10-030 freeze-dry system was used.

Plant tissue phosphorus determination. After plants were dried, the P content in the roots of each plant harvested was determined on a dry weight basis by the ammonium-molybdate method described previously (28).

Statistical analysis by standard error of the mean or least significant difference was conducted where appropriate.

RESULTS

Growth versus P depletion. Figure 1 shows the growth of phosphorus-supplemented and phosphorus-deprived root systems and the depletion of phosphorus from roots over time. The plants grown in a nutrient solution without phosphorus were quickly depleted of root P down to the critical level of 0.13% (10) within 4 weeks, whereas plants that received P maintained a somewhat constant root P concentration. These phosphorus-deprived plants also developed more extensive root systems as compared with the phosphorus-supplemented plants.

Influence of water potential on germination. In the presence of sorbitol the germination rate of *G. fasciculatus* spores peaked at from −4.0 to −6.0 bars and declined rapidly to almost no germination above and below this range (Fig. 2). Similar results were obtained in a replicate experiment.

Influence of root exudates on germination. The addition of root exudates, from plants grown in P-deficient and non-deficient solutions, to ROC agar had no significant effect on germination of *G. fasciculatus* spores (Table 1). The amount of germination was consistent across various concentrations and times of exudate collection. Similar results were obtained in a replicate experiment.

Hyphal elongation. Exudates from plants grown under P

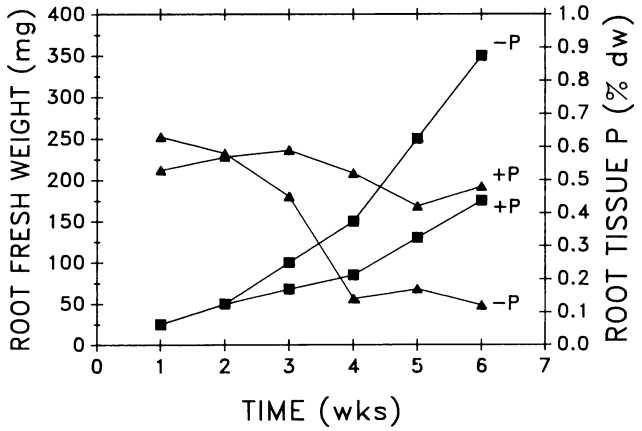

FIG. 1. Effect of phosphorus nutrition on *T. repens* root growth and root phosphorus depletion. Symbols: ■, total fresh weight of roots at the time of exudate collection; ▲, root tissue phosphorus on a percent dry weight (dw) basis. +P, With added phosphorus; −P, without added phosphorus. Root tissue phosphorus is expressed as the mean phosphorus level (percent dry weight of root tissue) of five root systems selected randomly from dishes each containing 50 plants. There was a significant difference in root P at weeks 4, 5, and 6 only.

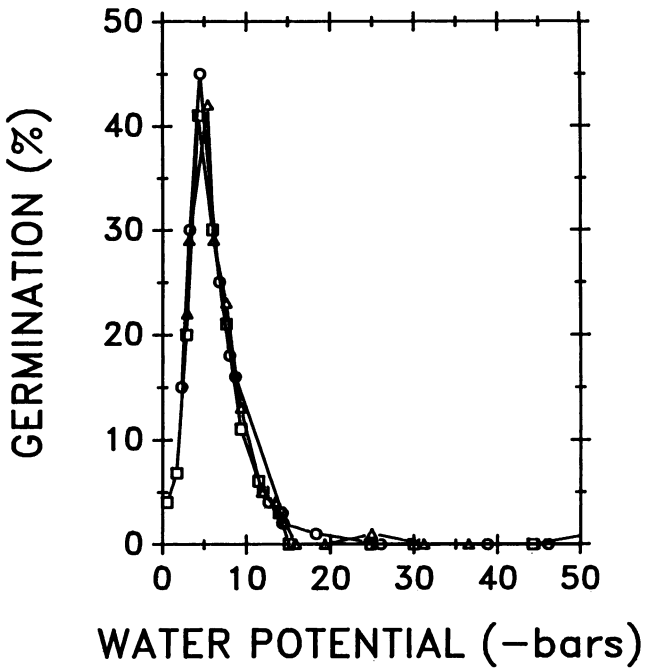

FIG. 2. Effect of substrate water potential as adjusted by sorbitol on germination of *G. fasciculatus* spores. Symbols: □; water agar; ○; VAM growth medium without added phosphorus; △; VAM growth medium with added phosphorus. Each point is the mean of five replicate plates each containing 20 spores.

deprivation stimulated greater hyphal elongation than exudates from plants grown with a P source (Fig. 3). Exudates collected at 2 weeks were more stimulatory to hyphal elongation than exudates collected at 4 or 6 weeks. The control treatments were not stimulatory to hyphal elongation.

TABLE 1. Influence of root exudates from *T. repens* on germination of *G. fasciculatus* spores on agar

Exudate solution added, ml[a]	% Germination		
	Week 2	Week 4	Week 6
−P			
0.0[b]	43[c]	43	43
0.0[d]	47	47	47
0.1	40	43	50
1.0	47	53	43
2.0	63	43	43
4.0	53	47	57
8.0	43	53	50
+P			
0.0	43	43	43
0.0	47	47	47
0.1	43	40	47
1.0	53	57	43
2.0	17	47	37
4.0	57	50	53
8.0	43	43	50

[a] Taken directly from staining jars containing 50 ml of sterile water in which 50 intact seedling root systems where allowed to exude for 24 h. Exudate solution from P-deficient plants (−P) and nondeficient plants (+P) was added to agar to bring it to 10 ml (total volume).
[b] Sterile water agar.
[c] Values represent the percentage of 30 spores that germinated.
[d] ROC agar.

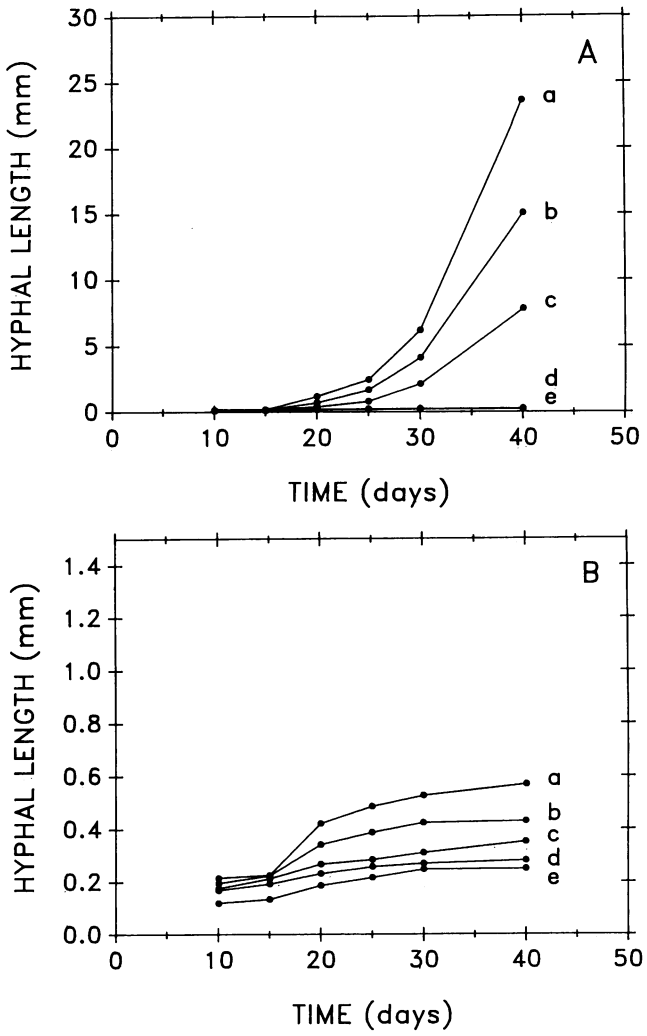

FIG. 3. Influence of exudates collected from 2-, 4-, and 6-week-old *T. repens* seedlings, (A) deprived of phosphorus and (B) supplied with phosphorus, on hyphal elongation of *G. fasciculatus* spores. Letters indicate exudates from (a) 2-week-old seedlings, (b) 4-week-old seedlings, (c) 6-week-old seedlings, (d) no exudates, and (e) water agar. Each point is the mean of all spores that germinated.

All exudate solutions more concentrated than 1.0 ml of exudate solution per 10 ml (total volume) of medium had no stimulatory effects on hyphal elongation. Only the 0.1- and 1.0-ml exudate amounts from P-deprived plants stimulated hyphal elongation, whereas the controls showed no effect (Table 2). Similar results were obtained in a replicate experiment.

Exudate quantification. The quantities of exudates from 2-, 4-, and 6-week-old seedlings grown with and without phosphorus were similar (Table 3).

An additional experiment incorporating lyophilized exudates (Table 3) from seedlings grown for 2 weeks with and without phosphorus was conducted. The exudates from the P-supplied and P-deprived plants were added to the same media used for previous experiments at a concentration of 8.3×10^{-5} g of exudate per ml of medium. This approximated the (0.1 ml) exudate concentrations from the 2-week-old plants used in the previous experiments. The hyphal

TABLE 2. Influence of root exudates from 2-, 4-, and 6-week-old *T. repens* seedlings on VAM hyphal elongation

Exudate solution added, ml[a]	Mean hyphal length[b] (mm) ± SEM		
	Week 2	Week 4	Week 6
−P			
0.1	21.42 + 1.93	14.72 + 1.03	7.62 + 0.37
1.0	11.68 + 0.92	8.62 + 1.15	4.26 + 0.74
2.0	0.55 + 0.24	0.64 + 0.31	0.86 + 0.24
4.0	0.44 + 0.13	0.42 + 0.13	0.47 + 0.13
8.0	0.37 + 0.08	0.31 + 0.10	0.34 + 0.13
+P			
0.1	0.57 + 0.26	0.43 + 0.30	0.35 + 0.40
1.0	0.47 + 0.20	0.45 + 0.10	0.23 + 0.10
2.0	0.39 + 0.17	0.40 + 0.13	0.27 + 0.14
4.0	0.31 + 0.07	0.31 + 0.10	0.34 + 0.23
8.0	0.23 + 0.11	0.22 + 0.08	0.18 + 0.14
Controls			
H_2O[c]	0.25 + 0.10		
No exudate[d]	0.28 + 0.20		

[a] Taken directly from staining jars containing 50 ml of sterile water in which 50 intact seedling root systems were allowed to incubate for 24 h. The exudate solution was reduced to 1/10 volume and added to agar to bring it to 10 ml (total volume). Abbreviations are as in Table 1.

[b] Values represent the mean hyphal length of germinated spores after 40 days.

[c] Sterile water agar.

[d] ROC agar.

growth stimulation with lyophilized exudates from P-deprived versus P-supplied plants was similar to what was achieved with nonlyophilized exudates (Fig. 3, Table 2). The experiment was conducted three times with similar results.

DISCUSSION

The inability of *G. fasciculatus* spores to germinate at high or low water potentials or correspondingly excessive or deficient moisture levels common in the soil environment could be important in the regulation of long-term survival. In addition, this optimum water potential range could be used in studies involved with the eventual culture of VAM fungi. It should be noted that sorbitol is not a substrate of VAM fungi. None of these observations on the influence of water availability on VAM germination is unexpected, since similar germination curves have been reported for many fungi (1, 9).

In this study, exudates, whether from phosphorus-deficient or nondeficient plants, had no effect on the germination of VAM fungal spores. The role of exudates has been recently discussed by Bowen (5, 6), who concluded that root exudates have little influence on the stimulation of VAM spore germination although they may have a role in other phases of the VAM development. Graham (12), in contrast, observed that exudates from both citrus and sudangrass had a stimulatory effect on VAM spore germination. However, the root exudates used were collected under nonsterile conditions and were a mixture of root exudates, microbes, and microbial metabolites. Thus, it is impossible to determine the origin of the compounds that did in fact stimulate spore germination.

Our study reveals a stimulatory influence of exudates from P-deficient clover seedlings as compared with those from P-supplemented seedlings on VAM fungal hyphal growth on agar. Our data also suggest that the quality of exudates from the root systems deprived of phosphorus may be responsible for this difference. This suggestion arises because exudate quantities did not differ betwen P-supplied and P-deprived seedlings (Table 3) and also because the hyphal growth stimulation associated with exudate incorporation from P-deficient plants was still present when equal amounts of lyophilized exudate from P-deprived and P-supplied plants were tested. Previously, however, Ratnayake et al. (31), Menge et al. (25), and Graham et al. (13) suggested that the quantity of exudates rather than a specific compound was responsible for the increased VAM infection. They also provided very strong evidence to support the theory that the decreased P level of plant roots is correlated with increased root membrane permeability and subsequent increased root exudation. This increased root exudation was suggested as the cause for increased VAM infection.

All of the previous studies concerning the influence of exudates on VAM were conducted with exudates collected at only one time, whereas our exudates were collected from the same plants over a 6-week period. This was done for a number of reasons. First, it has been reported (14, 34, 35) that increased plant age results in decreased exudation. Second, Schwab et al. (37) suggested that the P nutritional status of plants has very little influence on the quantity of root exudation and has no clear influence on the quality of root exudates. Hence, we felt that if an unknown factor were being produced by root systems deprived of phosphorus, which stimulates VAM formation, it would probably be exuded in the greatest quantities at very early stages in seedling growth and then would decrease with time. Production of this VAM formation factor at the earliest stage of seedling growth would potentially be the most advantageous to the plant since VAM benefits to the plant would be initiated quickly. This is also the time when VAM hyphal growth and infection usually increase rapidly. The data in Tables 2 and 3 and Fig. 3 support the hypothesis that an unknown VAM formation factor may be present and transient. Root exudates from 2-week-old P-deprived seedlings stimulated more hyphal growth than did root exudates from 4- or 6-week-old P-deprived seedlings. The data from Fig. 1 and Table 3 offer additional evidence for the existence of a VAM formation factor. At 2 weeks, the time of the first exudate collection, the tissue P levels in the root systems of plants grown with or without P were not significantly different; thus, their membrane permeabilities were probably not greatly different. In addition, at 2 weeks, the size of the root systems and the quantities of exudates between treat-

TABLE 3. Quantity of root exudates of *T. repens* at various seedling ages[a]

Treatment[a]	Seedling age (wk)	Total exudate collected (g)	Exudate per plant (g)	Exudate per plate (g)[b]
−P	2	2.08×10^{-2}	8.32×10^{-5}	8.32×10^{-4}
	4	0.78×10^{-2}	3.12×10^{-5}	3.12×10^{-4}
	6	0.43×10^{-2}	1.72×10^{-5}	1.72×10^{-4}
+P	2	2.13×10^{-2}	8.52×10^{-5}	8.52×10^{-4}
	4	1.03×10^{-2}	4.12×10^{-5}	4.12×10^{-4}
	6	0.44×10^{-2}	1.76×10^{-5}	1.76×10^{-4}

[a] Five staining jars (50 ml of solution per jar) were used for each treatment and seedling age; 50 plants were grown per jar, and exudates in solution from the five staining jars were pooled (250ml), lyophilized, and collected.

[b] Exudates from phosphorus-deficient (−P) and nondeficient (+P) plants.

[c] Calculated for exudate plate concentration of 1 ml exudate per 10 ml of medium.

ments were also similar. There was, however, a drastic difference in the effect that those exudates from P-deprived plants had on hyphal elongation as compared with exudates from nondeficient plants. Similarly, at 4 and 6 weeks, root systems of plants grown both with and without phosphorus grew larger, but the quantities of exudates changed very little between treatments. More importantly, even with a fourfold increase in the amount of root system from which exudates were collected, the influence on hyphal elongation decreased.

Plant growth curves and P depletion curves similar to those in Fig. 1 have been shown for other root systems (10). P-deficient plants generally produce a larger root system having greater surface area to explore a larger volume of soil in situ than other plants (8). In this study, it was not necessary for plants receiving P to make more roots since sufficient P was available. However, we would have expected the root systems to continue growing but at a slower pace. Possibly other environmental factors such as low light intensities were indirectly limiting root system growth.

In summary, because of the similarity of exudate quantities, the P depletion curves, the root system growth data, and the great differences in hyphal elongation between treatments, we suggest that it is the quality of exudates from plants experiencing P deprivation that is important in stimulating VAM hyphal growth.

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